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Angiogenic activity of sucupira (*Pterodon emarginatus*) oil

Atividade angiogênica do óleo de sucupira (Pterodon emarginatus)

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ABSTRACT

Aims: To assess the angiogenic activity of sucupira (*Pterodon emarginatus*) oil, popularly used as an antirheumatic, analgesic, antimicrobial, and anti-inflammatory agent.

Methods: The chick (*Gallus domesticus*) embryo chorioallantoic membrane (CAM) assay was used as an experimental model, incubated in a temperature- and humidity- controlled automatic incubator during the first five days. A circular hole was made at the wider base of the eggshell on the fifth day. After 13 days of incubation, filter paper discs containing sucupira oil and control substances were inserted into the CAM. Subsequently, the CAMs were removed and submitted to analysis and quantification of the vascular network. The nonparametric Kruskal-Wallis test, followed by Dunn's test, was used for the statistical analysis, and differences between the samples were set at a 5% level ($p<0.05$).

Results: Sucupira oil at a concentration of 1g/mL induced a significant increase in the percentage area of the CAM vascular network of the embryonated chicken eggs, when compared with the neutral control (water) and inhibitory control (dexamethasone) groups ($p<0.001$). However, no significant difference in the induction of the CAM vascular network ($p>0.05$) was demonstrated between the oil and the positive control (Biocure) groups.

Conclusions: The sucupira (*Pterodon emarginatus*) bean oil induced the formation of new blood vessels under the experimental conditions of the present study.

KEY WORDS: angiogenesis; membrane, chorioallantoic; *Pterodon emarginatus*

RESUMO

Objetivos: Avaliar a atividade angiogênica do óleo de sucupira (*Pterodon emarginatus*), popularmente utilizado como antirreumático, analgésico, antimicrobiano e anti-inflamatório.

Métodos: Foi aplicado como modelo experimental o ensaio na membrana corioalantóidea (MCA) de ovo embrionado de galinha (*Gallus domesticus*). Os ovos foram incubados em estufa automática com temperatura e umidade controladas durante os primeiros cinco dias. No quinto dia foi realizada uma abertura circular na base maior da casca do ovo. No 13º dia de incubação, discos de papel de filtro contendo óleo de sucupira e as substâncias controle foram inseridos sobre vasos da MCA. Posteriormente, as MCAs foram removidas e passaram por análise e quantificação da rede vascular. Para análise estatística foi utilizado o teste não paramétrico de Kruskal-Wallis, seguido de Dunn, sendo os valores aceitáveis para diferenças significativas entre as amostras de $p<0.05$.

Resultados: O óleo da fava de sucupira na concentração de 1g/mL induziu um aumento significativo na área de porcentagem da rede vascular na MCA do ovo embrionado de galinha, quando comparados aos grupos controles neutro (água) e inibidor (dexametasona) ($p<0.001$). Porém, não foi demonstrada diferença significativa entre o óleo e o controle positivo (Biocure) na indução da rede vascular na MCA ($p>0.05$).

Conclusões: O óleo da fava de sucupira (*Pterodon emarginatus*), nas condições deste experimento, foi responsável pela formação de novos vasos sanguíneos.

DESCRITORES: angiogênese; membrana corioalantóide; *Pterodon emarginatus*

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Abbreviations: CAM: chorioallantoic membrane; MCA: *membrana corioalantóidea*; NO nitric oxide; IL-1: interleukin-1; COX-2: enzyme cyclooxygenase-2; TNF-α: tumor necrosis factor alpha; ROS: reactive oxygen species

INTRODUCTION

Angiogenesis is defined as the formation of new blood vessels from preexisting capillaries, which involves a series of coordinated events: proliferation of endothelial cells, migration to distal sites, cell realignment, vessel formation, and production of a new membrane baseline [1,2].

Under normal circumstances, angiogenesis occurs during wound healing, in organ regeneration, in the female reproductive system during the maturation of the ovarian follicle, in *corpus luteum* formation, in regeneration of the endometrium after the menstrual cycle, in egg implantation, and in placenta formation. Moreover, the formation of new blood vessels is an important factor in a variety of pathological processes, such as tumor growth and metastasis, rheumatoid arthritis, diabetic retinopathy, psoriasis, obesity, atherosclerosis, ischemia, osteoporosis, among others [3-5].

Recently, emphasis has been given to the study of substances originating from plants that show biological activities capable of inducing the formation of new blood vessels, as their clinical application would be broad, including use in myocardial and central nervous system vascularization, after trauma or ischemia, in partial replacement of large-caliber arteries, and in wound healing [6-9].

The use of medicinal plants and their extracts has been documented for thousands of years, favoring prevention and treatment of disorders and dysfunctions of the human body [10,11]. Several plant species have shown biological activities capable of inducing angiogenesis, such as: in the latex from *Synadenium umbellatum*, "Cola-nota"; in the essential oil from *Schinus terebinthifolius* Raddi, "Aroeira"; in the oil from *Copaifera sp*, "Copaíba"; in the latex from *Hevea brasiliensis*, "Seringueira"; in the crude extract of the roots from *Memora nodosa*, "Silva manso"; and in the ethanolic extract from *Terminalia bellirica* [12-16].

In the Brazilian Cerrado biome, there are several commonly used plants with therapeutic potential, among which *Pterodon emarginatus* Vogel (*Pterodon pubescens* Benth) known as sucupira, sucupira-branca, or faveira, belonging to the family *Fabaceae* (*Leguminosae*) and to the subfamily *Faboideae*, stands out The seeds (beans) of the plant are used for medicinal purposes due to their antirheumatic, analgesic, anti-inflammatory, and antimicrobial properties as well as in the treatment of pharyngitis, bronchitis, and tonsillitis in humans [17-19].

Phytochemical studies using the genus *Pterodon* have shown the presence of alkaloid compounds and triterpenoids in the bark, diterpenoids and isoflavones in the seeds, isoflavones and triterpenoids in the stems, and β-sitosterol and stigmasterol in the leaves [19,23].

Given the highly popular use of sucupira oil and the presence of chemical compounds with biological activity in this plant, the aim of this study was to assess the angiogenic potential of the oil from *P. emarginatus*, using the chick embryo chorioallantoic membrane (CAM) assay.

METHODS

We used 40 fertilized eggs obtained from Rhoss chicken (*Gallus domesticus*) from the Department of Animal Science of the Pontifical Catholic University of Goiás. The sucupira oil was commercially purchased, manufactured by Amazon Leve®, Lot 010/12, at a concentration of 1 g/mL, in October 2012, with a shelf life of 2 years.

The following drugs and reagents were used in this study: sterile distilled water (Halex Istar Pharmaceutical Industry); dexamethasone (4 mg/mL; Aché Pharmaceutical Laboratories, Lot 2689); Latex Biomembrane (Biocure; Biotechnology "Pele Nova," Lot 04080100); 37% formaldehyde (Rioquimica Ltd., Lot 0402785); paraffin (Petrobras); and hematoxylin-eosin (HE) staining kit (Doles reagents).

To evaluate angiogenic activity, the CAM of embryonated chicken eggs was used and maintained in an automatic incubator under controlled temperature (38 °C) and humidity (65%). The eggs were shaken horizontally every 15 min during the first 5 days of incubation. In the study, the first day of incubation was considered the day of onset of embryonic development. At the end of this period, a circular hole (1.5 cm in diameter) was made with the aid of a micro-drill at the blunt end of the eggs, i.e., the region that contains the air sac. Upon completion of the hole, the shell membrane was removed, exposing the vascularized CAM. The hole was sealed with transparent adhesive tape and the egg was incubated again, without periodic shaking and with the holes turned upwards.

At the end of the 13th day of incubation, filter paper discs (0.5 cm ø), moistened with 2 µL of

sucupira oil (test), sterile distilled water (neutral control), 1% dexamethasone (inhibitory control), or Biocure biomembrane cutting (inductive control; with the same diameter as that of the filter paper discs) were placed directly on the membrane of 10 eggs per group, carefully avoiding the rupture and death of the embryo [12]. The eggs were returned to the incubator until day 16, when they were removed and the CAM was fixed in formaldehyde solution (10%) for 10 min [14].

CAM images were produced using a digital camera (Sony Cyber Shot 6.0 mega pixels) coupled to a light microscope, where the selected pixels were proportional to the degree of CAM vascularization, with an adopted resolution of 640 × 480 pixels and 24-bit RGB [24]. Subsequently, the newly formed CAM vascular network was quantified using the image processing *Gimp for Windows* program (version 2.0.5) for a better visualization of the blood vessels. Image analysis was performed using the *Image J* software (version 1.28), able to distinguish ranges of intensity level and thereby isolate and quantify pixels corresponding to the blood vessels.

The newly formed CAM vascular network was fixed in 10% formaldehyde solution and then embedded in paraffin. Each paraffin block was prepared and sectioned in a Spencer microtome at 5-µm thickness and then stained with hematoxylin-eosin to evaluate vascularization using a light microscope. Images were obtained using a digital camera connected to the microscope, with a Pinnacle Studio AV/DV Deluxe capture card.

Statistical analysis of the angiogenic activity of the latex of *P. emarginatus* was performed, comparing the percentage areas obtained from the CAM of treated and control groups. Differences between independent samples were compared using the nonparametric Kruskal-Wallis test, and Dunn's method was then used to compare the groups. The analyses were performed using the BioEstat software version 5.0 for Windows, and the acceptable p values for significant differences between the samples were $p < 0.05$ [25].

RESULTS

In the digital images obtained from the experiments, a larger network of newly formed vasculature was observed in the CAMs treated with sucupira oil (1g/ml) and with the positive control (Biocure) compared with the CAMs treated with neutral control (water) and inhibitor (dexamethasone) (**Figure 1**).

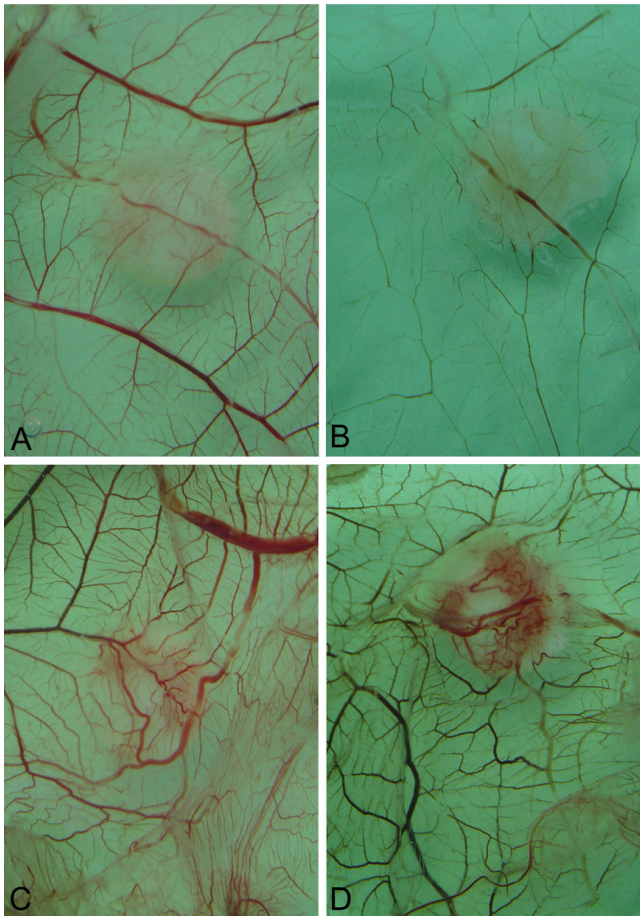

Figure 1. Vascular network formed in the chorioallantoic membrane (CAM) after treatment with "sucupira" oil along with controls. Neutral control; H_2O (**A**); Inhibitory control Dexamethasone (**B**); Positive control; Biocure (**C**); *Pterodon emarginatus* oil (**E**).

Subsequently, the quantification of blood vessel formation showed that treatment with sucupira oil at a concentration of 1 g/mL promoted a significant increase in the percentage area of the newly formed vascular network in the CAM of embryonated chicken eggs, when compared with the neutral control (water) and inhibitor (dexamethasone) groups ($p < 0.001$). However, no statistically significant difference was observed between the oil and the positive control group (Biocure) in the induction of the vascular network in the CAM ($p > 0.05$). Comparisons of percentage areas of the digital images of the different controls and sucupira oil are shown in **Table 1**.

The results of the histological analysis in the different controls and group treated with sucupira oil are consistent with the digital images and percentage of vascularization presented. The vascularization was minimal in the neutral group, and a clear inhibition of the blood vessels of the control inhibitor

Table 1. Percentage of vascularization obtained by treatment with sucupira oil and controls. All results were compared with the control groups by Kruskal-Wallis test ($p < 0.001$) followed by a multiple comparison.

No.	Positive Control Biocure	Neutral Control H_2O	Inhibitory Control Dexamethasone	Sucupira oil
1	58.4	34.3	15.9	40.6
2	49.7	35.2	14.7	45.9
3	45.1	27.7	10.2	42.3
4	53.5	26.4	10.8	47.6
5	56.7	27.5	12.1	58.7
6	43.1	26.9	12.4	53.8
7	59.3	27.8	9.5	41.4
8	57.9	33.1	12.9	47.2
9	55.6	32.3	9.3	53.6
10	44.6	40.4	12.2	49.5
Mean	*52.4	31.2	12.0	*48.1
Standard deviation	6.3	4.6	2.2	5.9

* No significant difference between groups ($p > 0.05$).

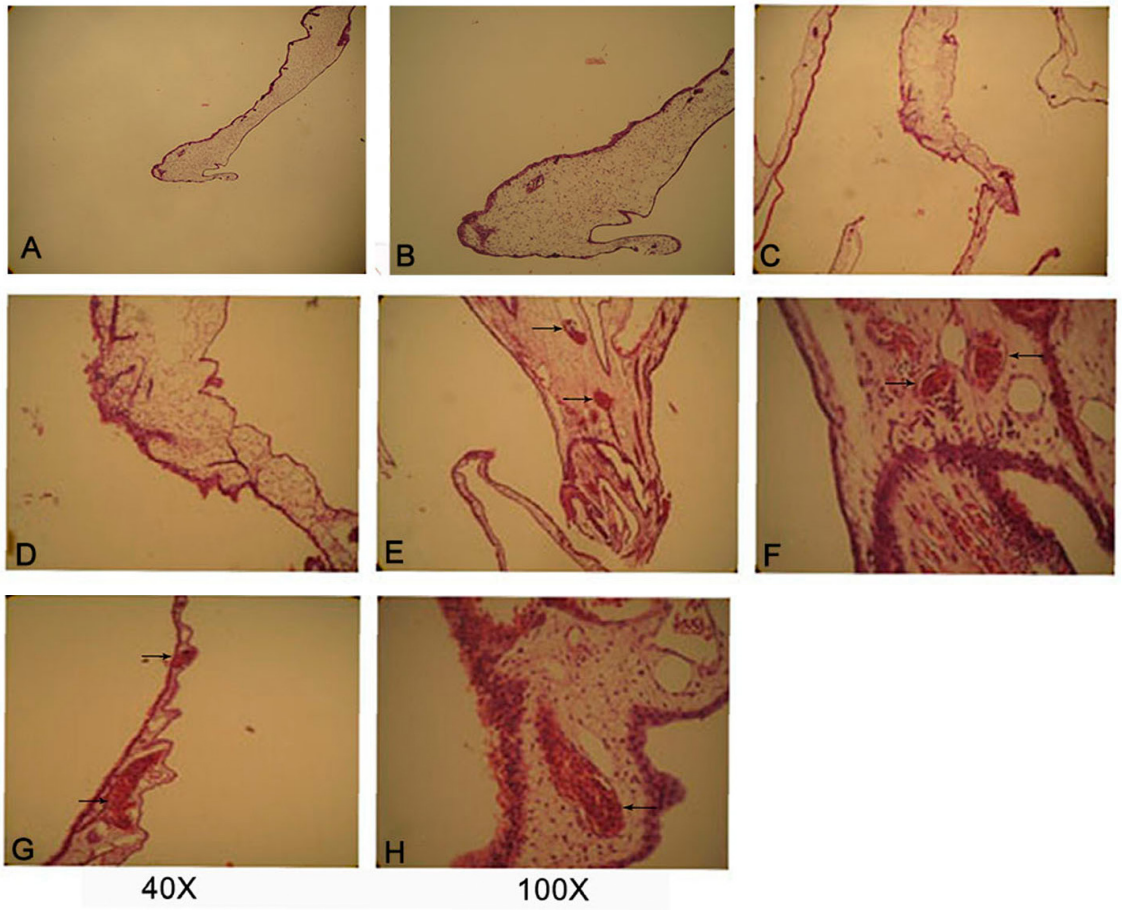

Figure 2. Histological sections stained with hematoxylin-eosin. Neutral control shows few blood vessel structures (**A**, **B**). Inhibitory control (dexamethasone) shows only connective tissue cells (**C** and **D**). Positive control (Biocure) shows some newly formed blood vessels and inflammatory cells (**E**) and in detail, the well-formed blood vessels and many nucleated erythrocytes (**F**). Treatment with "sucupira" oil results in well-organized vessels (**G**), packed with erythrocytes and inflammatory cells (**H**).

(dexamethasone) could be observed, since these areas displayed fewer and disorganized vascular endothelial cells. The positive control and sucupira oil-treated groups displayed a significant increase in newly formed vascular network, which appeared rich and organized, as well as in infiltrating inflammatory cells (**Figure 2**).

DISCUSSION

The results of this study demonstrate that *P. emarginatus* oil has angiogenic activity. The newly formed vascular network of the CAM (digital images and percentage of vascularization), as well as the histological analyses, confirm that treatment with *P. emarginatus* oil, at a concentration of 1 g/mL, stimulated the growth of new blood vessels in the CAM.

A CAM assay can be used as an "*in vivo*" model to study the angiogenic and antiangiogenic activity of a variety of substances, such as growth factors, cytokines, and hormones, as well as in tissue grafts and others. Drug toxicity can also be assessed in the CAM in terms of embryo death or adverse effects in the CAM, including inflammation and neovascularization [26].

The angiogenic activity of *P. emarginatus* oil is probably due to an activation of the inflammatory response. Physiological or pathological neovascularization is commonly associated with conditions in which different stages of infiltration of inflammatory cells occur. This response is essential to induce angiogenesis, tissue repair, skin wound healing, and other biological phenomena essential for the living being, in particular mammals [27].

In gastric ulcers and inflammatory diseases, the angiogenic effect is due to the modulation of inflammatory mediators, such as nitric oxide (NO) and interleukin 1 (IL-1), in which their reduced levels significantly inhibit the infiltration of leukocytes and neutrophils. This modulation arises from the action of terpenes present in *P. emarginatus* oil [28]. Moreover, it has been shown that ethylene oxide, a component of the oil of sucupira seeds, exhibits antiulcer activity in absolute ethanol, in addition to an anti-inflammatory effect on carrageenan-induced pleurisy and even an antitumor activity in some cell lines [29].

Experimental studies in mice indicate that the ethanol extract of *P. pubescens* Benth acts as an inhibitor of leukocyte proliferation, with a 58% reduction in the production of B cells and 89% reduction in T cells, and *in vitro*, being touted as a controller of an exaggerated cellular immune response in inflammatory diseases [30].

Other experiments in mice show that the terpene compounds of the crude hexane extract of *P. emarginatus* inhibit the oxidative and nitrosative stress induced by acute exercise, leading to an antioxidant effect and anti-inflammatory action [31,32].

However, even considering the results of this experiment, which demonstrated the angiogenic effect of sucupira, other studies also indicate that a dose of 300 mg/kg of oil from this plant shows an antiproliferative activity in cultured tumor cells. This could be due to the action of isoflavones and diterpenes that act by inhibiting the enzyme cyclooxygenase-2 (COX-2), which is responsible for the inflammatory process [33]. Therefore, the stimulation of some pro-inflammatory cytokines [IL-1 and tumor necrosis factor alpha (TNF-α)] in various cell types, including endothelial cells, promotes the expression of COX-2 [34].

There are also reports of certain structurally related flavonoids, such as 3-hydroxyflavone, 3',4' and 2',3'-dihydroxyflavone, rutin, kaempferol, fisetin, apigenin, and luteolin inhibiting the proliferation of normal and tumor cells and, "*in vitro*", angiogenesis [35,36]. A recent CAM assay using the flavonoid derivative R24 found that this compound inhibited neovascular formation in cell lines of lung, pancreatic, colorectal, and prostate cancers, in addition to promoting the generation of reactive oxygen species (ROS), depending on the dose applied [35].

Therefore, because angiogenesis is a process related to cell proliferation, it is important to note that different concentrations of sucupira oil tested in numerous studies are diversified, which has allowed the observation of opposite effects: the potential angiogenic effect of this plant oil by the induction of an inflammatory and antiangiogenic response owing to its toxicity in cancerous cells.

According to the data presented in this study, it was concluded that sucupira oil showed angiogenic activity. However, other studies demonstrate the antiangiogenic and toxic effect of this oil when used at concentrations different from those used in this study. Moreover, common compounds present in several plants have demonstrated different effects, suggesting that interactions with other elements yield different results, which is shown by the study of the active ingredients of individual plants.

Further investigations are required to elucidate the angiogenic activity of sucupira oil, with the aim of observing not only the angiogenic effect of the overall composition of the oil, but also that of its individual active ingredients.

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